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Cell-free pool of CD14 mediates activation of transcription factor NF-κB by lipopolysaccharide in human endothelial cells

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ABSTRACT Lipopolysaccharide (LPS), a major envelope component of Gram-negative bacteria, is the most frequent causative agent of septic shock and disseminated intravascular coagulation. LPS activates both CD14-positive (monocytes, macrophages, polymorphonuclear leukocytes) and CD14-negative (B-cell lines, endothelial cells) cells. CD14, a 55-kDa glycosyl-phosphatidylinositol-anchored membrane protein present on mature myeloid cells, serves as a receptor for LPS in complex with a soluble (serum-derived) LPS-binding protein (LBP). In this report, we show that human umbilical vein endothelial cells (HUVEC), which do not express measurable CD14 protein, become 3000-fold more sensitive to LPS-induced activation in the presence of serum, as measured by activation of the transcription factor NF-κB and expression of mRNA encoding tissue factor, a procoagulant molecule. This enhanced responsiveness of HUVEC is specifically mediated by the cell-free pool of CD14 (soluble CD14, sCD14) found in serum. The role of sCD14 in HUVEC activation by LPS was established by (*i*) the blocking effect of monoclonal anti-CD14 antibodies which discriminate between cell-bound and sCD14, (*ii*) the lack of the serum-enhancing effect after immunodepletion of sCD14, and (*iii*) establishing a reconstituted system in which recombinant sCD14 was sufficient to enhance the effects of LPS in the absence of serum and without a requirement for LBP. Thus, this mechanism of endothelial cell activation by LPS involves a cell-free pool of sCD14 most likely shed from CD14-positive cells of the monocytic lineage.

Lipopolysaccharide (LPS)-producing Gram-negative bacteria are the most frequent cause of septic shock, which affects approximately 400,000 patients annually in the United States (1). Vascular complications of septic shock, including hypotension and disseminated intravascular coagulation, are related to endothelial injury induced by LPS (2). Recently, a 55-kDa glycoprotein termed CD14 and expressed through a glycosyl-phosphatidyl inositol anchor on the surface of monocytes, macrophages, and polymorphonuclear leukocytes (3) was established as a cellular receptor for LPS (4, 5). Complexes of LPS and the serum-derived LPS-binding protein (LBP) have been shown to potently stimulate tumor necrosis factor α (TNFα) production by monocytes and macrophages in a CD14-dependent manner (4, 6). However, certain CD14-negative cells, including vascular endothelial cells and B lymphocyte cell lines, are also responsive to LPS (7, 8).

Endothelial cells stimulated with LPS and the inflammatory cytokines TNFα and interleukin (IL)-1 exhibit an activated phenotype due to the expression of genes encoding tissue factor, plasminogen activator inhibitor 1, and leukocyte adhesion molecules, as well as the release of soluble cytokines, including IL-1, IL-6, IL-8, monocyte chemoattractant protein, and the colony stimulating factors (CSFs) (ref. 7 and references therein). The response of endothelial cells to LPS is enhanced by serum (9–12). Recently, a role for soluble CD14 (sCD14) present in serum was demonstrated in LPS-induced cytotoxicity to bovine endothelial cells and expression of endothelial leukocyte adhesion molecule (E-selectin) in human endothelial cells (13). Although LPS can bind to sCD14 (14), alterations in intracellular signaling pathways induced by this complex are unknown. Moreover, the role sCD14 plays in LPS-induced signal transduction events required for altered gene transcription has not been previously addressed.

In this regard, it is well recognized that LPS is a potent activator of the transcription factor NF-κB/Rel family of DNA-binding proteins (15). Several LPS-inducible genes—including tissue factor (16), IL-1 (17), IL-6 (18), IL-8 (19), granulocyte colony-stimulating factor (G-CSF) (20), vascular cell adhesion molecule 1 (VCAM-1) (21–23), and E-selectin (24–26)—contain elements within their promoter regions that are recognized by one or more members of the NF-κB/Rel transcription factor family. Whereas the signal transduction pathway(s) involved in the activation of NF-κB in endothelial cells is unknown, recent studies with THP-1 monocytic cells indicate that LPS stimulates phosphorylation of MAD3, termed IκBα, an inhibitor of NF-κB (27). Phosphorylation of MAD3 regulates translocation of NF-κB from the cytoplasm to the nucleus (28). Together, these findings suggest that LPS-induced activation of NF-κB in endothelial cells may play a pivotal role in increasing expression of multiple gene products which ultimately contribute to endothelial activation and vascular injury.

In the present study, we measured the activation and nuclear translocation of NF-κB DNA-binding activity by gel mobility shift analysis as a specific index of the endothelial response to LPS. We report here that primary cultures of human umbilical vein endothelial cells (HUVEC) respond to ng/ml concentrations of LPS in a serum-dependent manner. We further demonstrate that a cell-free pool of sCD14 is the active serum factor responsible for the enhanced sensitivity of HUVEC to LPS. Both activation of NF-κB and increased mRNA encoding tissue factor are induced in endothelial cells after exposure to ng/ml concentrations of LPS in the presence of sCD14. Significantly, sCD14 has the capacity to enhance these effects of LPS on human endothelial cells independent of LBP.

MATERIALS AND METHODS

Culture and Treatment of Cells. Primary cultures of human endothelial cells obtained from collagenase-digested umbilical veins were established in M199 medium with 20% fetal

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Abbreviations: LPS, lipopolysaccharide; LBP, LPS-binding protein; HUVEC, human umbilical vein endothelial cells; sCD14, soluble CD14; TNFα, tumor necrosis factor α; IL, interleukin; CSFs, colony stimulating factors; E-selectin, endothelial leukocyte adhesion molecule-1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

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bovine serum (ICN-Flow, Costa Mesa, CA), porcine intestinal heparin (Sigma) at 100 μg/ml, endothelial mitogen (Biomedical Technologies, Stoughton, MA) at 50 μg/ml, penicillin at 50 units/ml and streptomycin (ICN-Flow) at 50 μg/ml, and 25 mM Hepes (ICN-Flow) in gelatin-coated plates (29, 56). Confluent monolayers (3–5 × 10^5 cells, passages 2–4) were rinsed twice with Hanks' balanced salt solution and exposed to *Escherichia coli* LPS (O127:B8, Difco) for 2 hr in serum-free medium (Endothelial SFM, GIBCO/BRL) with and without the addition of 10% human serum containing no detectable LPS (North American Biologicals, Miami). Mono Mac 6 cells were cultured in RPMI 1640 medium with 10% fetal bovine serum, 0.1 mM nonessential amino acids (GIBCO), 1 mM oxaloacetic acid, 1 mM sodium pyruvate, insulin (Sigma) at 9 μg/ml, penicillin at 50 units/ml, and streptomycin at 50 μg/ml (30).

Nuclear Extracts. Following experimental treatment of endothelial cells, nuclear extracts were prepared as described by Molitor *et al.* (31) with the following modifications. Cell monolayers (3–5 × 10^6 cells) were harvested, washed in cold phosphate-buffered saline (PBS) (57), and incubated in 80 μl of buffer A (10 mM Hepes, pH 8.0/1.5 mM $MgCl_2$/10 mM KCl/0.5 mM dithiothreitol/200 mM sucrose/0.5 mM phenylmethanesulfonyl fluoride/0.5 μg of leupeptin per ml/0.5 μg of aprotinin per ml/0.5% Nonidet P-40) for 15 min at 4°C. Nuclei liberated by the lysis step were collected by microcentrifugation, rinsed once in buffer A, and resuspended in 80 μl of buffer B [20 mM Hepes, pH 8.0/20% (vol/vol) glycerol/0.1 M KCl/0.2 mM EDTA/0.5 mM phenylmethanesulfonyl fluoride/0.5 mM dithiothreitol/0.5 μg of leupeptin per ml/0.5 μg of aprotinin per ml]. Nuclei were sonicated for 10 s at 15% output (Virsonic Cell Disrupter 475, VirTis) and clarified by microcentrifugation for 30 s. The resulting supernatants contained 1–2 mg of protein per ml by Bradford assay (32) with bovine serum albumin as the standard. Nuclear extracts were frozen on dry ice and stored at −80°C.

Electrophoretic Mobility Shift Assay. A double-stranded oligonucleotide containing the NF-κB binding site from the murine immunoglobulin κ enhancer (5′-AGCTTAGAGGGGACTTTCCGAGAGGA-3′) was labeled with 50 μCi (1 Ci = 37 GBq) of [α-^{32}P]dATP (New England Nuclear) and the Klenow fragment of *E. coli* DNA polymerase I. Binding reactions were in 20 μl containing 15 μg of nuclear extract protein, binding buffer [10 mM Tris·HCl, pH 7.5/20 mM NaCl, 1 mM dithiothreitol/1 mM EDTA/5% (vol/vol) glycerol/1 μg of poly(dI-dC)/1 μg of single-stranded salmon testis DNA], and 50,000 cpm of ^{32}P-labeled DNA. Reaction mixtures were incubated at room temperature for 20 min and analyzed by electrophoresis on a nondenaturing 4% polyacrylamide gel at 180 V for 2 h under the high-ionic-strength conditions described by Ausubel *et al.* (ref. 33, pp. 12.2.1–12.2.10). After electrophoresis, gels were dried and DNA–protein complexes were localized by autoradiography for 18 h.

Blocking of CD14 with Monoclonal Antibodies. Murine monoclonal antibodies specific for recombinant human sCD14 (5G3, 18E12, 26F3, 28C5, and 10B7) were generously provided by Ann Moriarty and Didier Leturcq (R. W. Johnston, San Diego, CA) (34). Endothelial SFM containing 10% human serum was incubated with monoclonal antibody (IgG, 10 μg/ml) for 15 min at 37°C prior to the addition of LPS at 100 ng/ml. HUVEC were washed twice with Hanks' balanced salt solution and incubated with control or antibody-treated medium containing LPS for 2 h prior to preparation of nuclear extracts.

Immunodepletion of Human Serum with Anti-CD14. Anti-CD14 (MY4) and an isotype control IgG2b (Coulter) were coupled to protein G-agarose (Calbiochem) in 0.1 M sodium acetate, pH 5, at 4°C. Antibody-coupled agarose was rinsed extensively in Hanks' balanced salt solution and incubated with human serum overnight at 4°C. After centrifugation to remove antibody-coupled agarose, serum was tested by Western blotting with anti-CD14 and the ECL chemiluminescence detection system (Amersham) to confirm that CD14 was depleted.

Northern Analysis. mRNA from 1 × 10^7 cells was isolated using the RNAgents total RNA and PolyATract mRNA isolation systems (Promega). mRNA was separated by electrophoresis on a 1% agarose/formaldehyde gel, transferred to a Hybond-N membrane (Amersham), and immobilized by UV irradiation with a Bioslink Transilluminator (Bios, New Haven, CT). Blots were prehybridized for 6 h and hybridized overnight (ref. 33, p. 4.9.7) at 42°C with ^{32}P-labeled probes (Stratagene Prime-it II kit). cDNA clones for CD14 and tissue factor were generously provided by Brian Seed (Massachusetts General Hospital) and Nigel Mackman (The Scripps Research Institute), respectively. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probe was obtained from Clontech.

Reagents. Recombinant sCD14 produced in CHO cells and purified human LBP (35) were provided by Ann Moriarty and Didier Leturcq. All other reagents were cell culture or molecular biology grade from Sigma.

RESULTS

Activation of NF-κB by LPS Is Dependent upon Serum. Early passages (2–4) of HUVEC derived from primary cultures were grown in the presence of fetal bovine serum containing no detectable LPS. When such cultures were incubated in a serum-free medium they exhibited a striking dependence on human serum for their responsiveness to LPS, as measured by activation of NF-κB DNA binding in nuclear extracts (Fig. 1). In the absence of human serum, concentrations of LPS up to 30 μg/ml induced only weak activation of NF-κB in HUVEC. In the presence of 10% human serum, LPS at 10 ng/ml elicited measurable NF-κB activation (Fig. 1). Thus, the addition of human serum enhanced the sensitivity of HUVEC to LPS 3000-fold (compare lanes 5 and 8). In sharp contrast, TNFα and phorbol 12-myristate 13-acetate, which also potently activate NF-κB in HUVEC, did not require serum for their effect (data not shown).

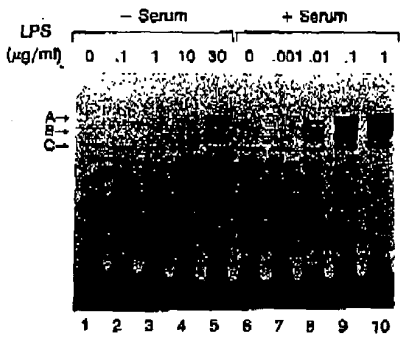

FIG. 1. LPS activation of NF-κB DNA binding in HUVEC is dependent on serum. HUVEC were treated with indicated concentrations of LPS for 2 h in the absence and presence of 10% human serum. Nuclear extracts were prepared and assayed for NF-κB DNA-binding activity by gel retardation analysis. Arrows indicate position of three DNA-binding complexes. Formation of complexes A and B was decreased by competition with unlabeled wild-type but not mutant NF-κB oligonucleotides (not shown), verifying the specificity of binding. Complex C is constitutively present and binds nonspecifically. The data shown are representative of three experiments.

LPS Activation of HUVEC Is Blocked by Anti-CD14 Antibodies. Previous studies have shown that treatment of CD14-positive monocytic cells with anti-CD14 antibodies markedly inhibits their response to LPS (4, 34). Among a panel of five monoclonal antibodies raised against recombinant CD14, we found that three (18E12, 26F3, and 28C5) strongly inhibited LPS-induced expression of nuclear NF-κB in HUVEC in the presence of 10% human serum (Fig. 2*A*). Two of these antibodies (28C5 and 18E12) also inhibited NF-κB activation in response to LPS in the human monocytic cell line Mono Mac 6, which is CD14 positive (30) (data not shown). Surprisingly, monoclonal antibody 26F3 was inhibitory with HUVEC (Fig. 2*A*, lane 8), but it failed to inhibit NF-κB activation by LPS in Mono Mac 6 cells (data not shown). Notably, this particular antibody recognizes the soluble form of CD14 but not cell membrane-anchored form (Didier Lenoir, personal communication). Furthermore, serum which was depleted of sCD14 did not render HUVEC responsive to LPS (Fig. 2*B*). These results suggest that sCD14 rather than the membrane-anchored form mediates the activation of intracellular signaling events by LPS in HUVEC.

Because anti-CD14 monoclonal antibodies ablated the serum-dependent responsiveness of HUVEC to LPS, we examined expression of CD14 in HUVEC. Consistent with prior reports (36), no evidence was obtained for the presence of either cell surface or cytosolic CD14 by flow cytometry analysis and immunohistochemical staining with HUVEC, using Mono Mac 6 cells as a positive control (data not shown). We further examined CD14 expression by evaluating steady-state CD14 mRNA levels in HUVEC. As shown in Fig. 3, Northern blots confirmed that HUVEC expressed low levels of CD14 mRNA relative to Mono Mac 6 cells, and treatment with LPS failed to augment the level of CD14 mRNA expression in either cell type. Cumulatively, these data indicate that human endothelial cells do not express membrane-bound CD14, but instead require a cell-free pool of CD14 present in serum in order to respond to LPS.

sCD14 Renders HUVEC Responsive to LPS in Serum-Free Medium. To examine whether cell-free CD14 and/or LBP could potentially replace serum as a mediator of LPS responsiveness in HUVEC, we established a reconstituted system containing recombinant cell sCD14 and LBP purified from human serum (35). Both were used in concentrations equivalent to those found in the 10% human serum used in this study (CD14 at 0.1 μg/ml and LBP at 1 μg/ml). As shown in Fig. 4, LPS incubated with LBP alone caused only minimal stimulation of HUVEC in serum-free medium, as measured by nuclear translocation of NF-κB. On the other hand, LPS in the presence of sCD14 was effective in activating NF-κB in HUVEC. The addition of LBP to sCD14 did not further enhance the response to LPS above that obtained with sCD14 and LPS. To confirm that serum-derived proteins such as LBP were not contributing to the observed effect of LPS and sCD14, cells were placed in serum-free medium for 16 h prior to stimulation. Similar to cells that had been washed and placed immediately in serum-free medium, these serum-starved cells responded to LPS when sCD14 was added (not shown).

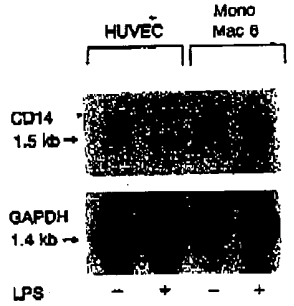

FIG. 3. Northern analysis of mRNA encoding CD14 from HUVEC and Mono Mac 6 cells. mRNA was isolated from cells exposed to LPS (100 ng/ml) for 0 and 3 h and analyzed. GAPDH mRNA was used as the internal control. The blot represents one of two independently performed experiments.

To determine whether the intracellular signals generated by LPS/sCD14 were sufficient to induce a known LPS-responsive gene, we examined expression of tissue factor mRNA, whose expression is stimulated by LPS in HUVEC (37). When HUVEC were stimulated with LPS and sCD14 in serum-free medium, steady-state expression of tissue factor mRNA was observed (Fig. 4*B*). Thus, in keeping with the observed dependence of LPS-mediated NF-κB activation on sCD14, increased levels of tissue factor mRNA were induced by LPS and sCD14 in the absence of LBP or other serum factors.

DISCUSSION

In this study, we demonstrate that primary cultures of HUVEC, which do not express detectable CD14 on their surface, respond to ng/ml concentrations of LPS in a serum-dependent manner, as determined by measuring activation of the transcription factor NF-κB. The factor responsible for the serum-dependent effect was identified as the soluble form of CD14, on the basis of the following criteria. First, the enhancing effect of serum was completely blocked by three

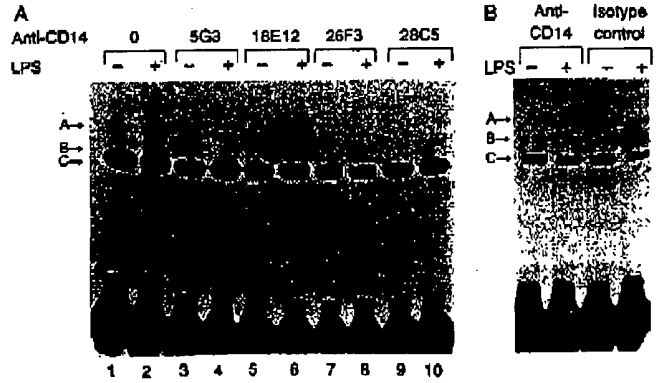

FIG. 2. Monoclonal antibodies to CD14 block LPS-induced activation of NF-κB DNA binding in HUVEC. (*A*) HUVEC were exposed to LPS (100 ng/ml) for 2 h in media containing 10% human serum which had been pretreated with or without the indicated monoclonal antibodies at 10 μg/ml. An additional monoclonal anti-CD14, 10B6, also was evaluated in this assay and did not inhibit activation of NF-κB in HUVEC (not shown). (*B*) HUVEC were exposed to LPS (100 ng/ml) for 2 h in the presence of 10% human serum which had been treated with anti-CD14 (My4) or an isotype-matched control antibody coupled to protein G agarose. The data shown are representative of two experiments.

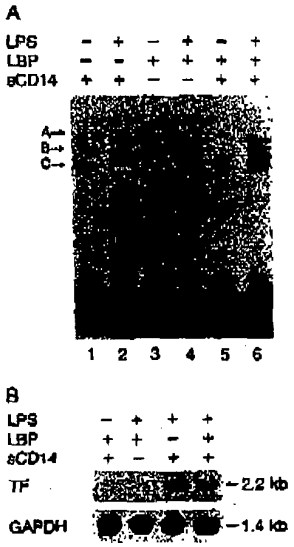

FIG. 4. Recombinant sCD14 renders HUVEC responsive to LPS in serum-free medium (SFM). HUVEC were exposed to LPS (100 ng/ml) in SFM for 2 h (*A*) or 3 h (*B*) in the absence or presence of sCD14 (0.1 μg/ml) and/or LBP (1 μg/ml). (*A*) Nuclear extracts were prepared and assayed for NF-κB DNA binding. (*B*) mRNA was prepared and probed for tissue factor (TF) or GAPDH as an internal control. The blot represents one of two independently performed experiments.

monoclonal antibodies to CD14, including one which recognized soluble but not cell membrane-bound CD14. Second, the selective removal of sCD14 from serum by immunodepletion abolished the serum-enhancing effect on LPS-mediated activation of HUVEC. Third, in reconstitution experiments, recombinant sCD14 could replace serum without further supplementation with LBP, an additional serum factor which can complex with LPS (6). In a reconstituted system the concentration of recombinant sCD14 needed for LPS-induced activation of NF-κB was 0.01 μg/ml (unpublished observations), while that found in 10% human serum is 0.1 μg/ml. It is noteworthy that in our serum-free system, treatment of HUVEC with LPS and LBP, but without sCD14, did not lead to activation of NF-κB or to an increase in tissue factor mRNA. Thus, serum-derived LBP is apparently dispensable in the response of HUVEC to LPS. Together, these data show that a cell-free soluble form of CD14 is responsible for the serum-dependent effect of LPS, via a molecular mechanism of endothelial cell activation that does not require serum-derived LBP.

Serum dependence in the response of bovine and human endothelial cells to LPS has been the subject of a number of investigations (9–13). Most recently, the cytotoxic effects of LPS towards bovine brain microvascular endothelial cells were linked to the presence of serum-derived sCD14 (13). In addition, LPS-induced expression of E-selectin in human endothelial cells was attributed to sCD14 (13). The results described herein extend these observations to the pathway of activation of the transcription factor NF-κB and expression of mRNA encoding tissue factor in human endothelial cells. The complexes formed between the radiolabeled double-stranded oligonucleotide probe containing the NF-κB binding site used in these experiments and nuclear proteins were similar to those obtained by using the oligonucleotide probe representing the NF-κB binding site present in other LPS-induced genes such as the urokinase-type plasminogen activator gene (results not shown). However, activation of NF-κB is necessary but not sufficient for induction of a number of LPS-responsive genes, including E-selectin and tissue factor (16, 26). Induction of E-selectin expression is primarily regulated at the transcriptional level (25, 26), whereas in the case of tissue factor, the response to LPS involves increased mRNA synthesis as well as posttranscriptional regulation (37, 38). Thus, data presented here and in previous reports suggest that sCD14-enhanced LPS stimulation can trigger multiple signaling pathways which result in endothelial cell activation.

These results raise the possibility that cell-free sCD14 present in the blood may prime LPS for enhanced ligation with a putative LPS receptor on HUVEC. It has been demonstrated that sCD14 can form stable complexes with LPS *in vitro* (14). The mechanism of enhancement of LPS interaction with human endothelial cells by sCD14 remains unresolved. Evidence for an LPS receptor on LPS-sensitive cells which lack CD14 has emerged from studies with the pre-B-cell line 70Z/3 (39). However, this putative receptor has not been directly linked to LPS-induced signal transduction within the intact cell. Additional evidence for an LPS receptor has been obtained in endothelial cells, 70Z/3 cells, and monocytic cells, where activation by LPS is inhibited by nontoxic forms of LPS and nontoxic derivatives of lipid A, the active moiety of LPS (40–47). These studies suggest that the nontoxic compounds inhibit cellular responses to LPS by blocking receptor-mediated recognition of LPS/lipid A on the cell surface. Recently, 70Z/3 cells transfected with CD14 cDNA were shown to express the glycosyl-phosphatidylinositol-linked membrane form of CD14 and were 10,000-fold more sensitive to LPS than mock-transfected cells (8). Consistent with our findings in HUVEC, increased sensitivity of the CD14-transfected 70Z/3 cells was observed in the absence of LBP. Thus, either sCD14 or cell membrane CD14 may facilitate recognition of LPS by a second, signal-generating, receptor which is more responsive to LPS when the LPS is presented in the context of CD14.

In monocytic cells, human immunodeficiency virus type 1 (HIV-1) gene expression is regulated by factors which bind the NF-κB DNA element (48). A recent study demonstrated that expression of CD14 was necessary for LPS-induced HIV-1 production in certain monocytic cell lines (49). Since human liver sinusoidal endothelial cells can be readily infected with HIV-1 *in vitro* (50), it will be important to establish whether sCD14 plays a role in LPS-induced activation of HIV-1 gene expression in virus-infected endothelial cells.

Although data presented here and elsewhere clearly demonstrate that sCD14 enhances LPS responsiveness of endothelial cells *in vitro*, the source of sCD14 *in vivo* remains to be established. The cell-free pool of soluble CD14 in blood may be derived from monocytic cells which shed CD14 from their surface (51). While the physiologic mechanism of shedding of CD14 has not been elucidated, it may involve cleavage by both proteolysis and phosphatidylinositol-specific phospholipase C (52). Since levels of CD14 are increased in blood of septic patients (53), shedding of CD14 from monocytic cells may set the stage for endothelial cells to respond to low levels of LPS in the circulation (54, 55). The role of sCD14 in enhancing vascular injury in LPS-induced generalized Shwartzman reaction (2) warrants consideration. The initial dose of LPS may result in increased shedding of sCD14 from monocytic cells, rendering endothelial cells susceptible to subsequent challenge by LPS. Activation of endothelium by cytokines, including TNFα and IL-1 (7), as well as the direct effects of LPS enhanced by sCD14, may result in more profound changes in vascular endothelium. This mechanism of sCD14-dependent endothelial cell activation by LPS may

therefore play a central role in enhancing the vascular complications of Gram-negative bacteremia such as septic shock and disseminated intravascular coagulation.

Note Added in Proof. After submission of this paper Pugin *et al.* (58) reported that LPS-induced expression of vascular cell adhesion molecule 1 and IL-8 in human endothelial cells is mediated by LPS-binding protein and soluble CD14.

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